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GRANT NUMBER DAMD17-97-1-7335

TITLE: Early Changes in Apoptosis and Proliferation to Predict Response and Resistance to Chemotherapy in the Treatment of Breast Cancer

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REPORT DATE: September 1998

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE			Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.				
1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE September 1998		3. REPORT TYPE AND DATES COVERED Annual (1 Sep 97 - 31 Aug 98)
4. TITLE AND SUBTITLE Early Changes in Apoptosis and Proliferation to Predict Response and Resistance to Chemotherapy in the Treatment of Breast Cancer				5. FUNDING NUMBERS DAMD17-97-1-7335
6. AUTHOR(S) Dowsett, Mitchell, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Royal Marsden Hospital London, SW3 6JB England				8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSORING / MONITORING AGENCY REPORT NUMBER
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited				12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 words) The project has 2 interconnecting aims: (i) to confirm and extend the observations, that apoptosis is increased and proliferation is decreased in primary breast cancer shortly after chemo and endocrine therapy, such that the predictive power of these changes for clinical response can be assessed, (ii) to develop an automated method for analysing apoptosis in fine needle aspirates (FNAs) taken from breast carcinomas. Aim (i): we have confirmed that apoptosis significantly increases 24 hours after starting chemotherapy and demonstrated that proliferation also falls by a mean of approximately 30% at this time. We have shown that significant changes in proliferation after 21 days chemo, endocrine- or chemoendocrine-therapy occur only in groups responding to treatment. Predictive power remains to be assessed. Aim (ii): initially encouraging findings from the application of flow cytometry to apoptotic measurements in FNAs involved the derivation of a statistical approach to the definition of the apoptotic population. The measurements correlated significantly with those made by conventional techniques on tissue sections. Further work indicates that flow cytometry is unlikely reliably to separate the apoptotic population but our preliminary work with Laser Scanning Cytometry indicates that this approach should be applicable to FNAs.				
14. SUBJECT TERMS Breast Cancer, apoptosis, proliferation, response, resistance, chemotherapy.				15. NUMBER OF PAGES 16
				16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

FOREWORD

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INTRODUCTION:

Preoperative or so-called neoadjuvant medical therapy provides a unique setting for the "bioclinical" study of breast cancer. Biological determinants of response to therapy may be studied in the tumour and related to clinical response in the same lesion. These studies may be made by radiological approaches such as magnetic resonance spectroscopy or positron emission tomography but most data, including ours, have been derived from immunocytochemical pathological studies of small tissue biopsies in core cuts or fine needle aspirates.

The grant proposal for this work (in late 1996) was made shortly after our finding that significant increases in apoptosis occurred within 24 hours of starting chemotherapy (Ellis et al, 1997). We had also conducted reproducibility studies using 14-gauge core-cut biopsies which indicated that at least 50% changes in apoptosis or proliferation (as measured by Ki67 staining) were required for changes in an individual tumour to be significant with 95% confidence (Ellis et al, 1998). In this original study a trend to a downward change in proliferation after 24 hours chemotherapy was not statistically significant. We also knew that significantly reduced proliferation in patients on hormonal therapy occurred in the first few weeks of treatment (DeFriend et al, 1994; Clarke et al, 1993) but the relationship with clinical response was unclear. We therefore wished to address the possibility that these early changes in the key determinants of tumour growth, which predate measurable clinical response, could be used to predict response and optimise patient therapy.

Our programme of work has focused on

- (i) (a) the confirmation that apoptosis is increased after 24 hours and elucidation of the proportion of patients in which this occurs.
- (b) establishment of whether the trend to reduced proliferation at 24 hours is significant in a larger population.
- (ii) assessment of whether the changes in proliferation after 2-3 weeks endocrine-, chemo- or chemoendocrine-therapy are associated with response.
- (iii) derivation of an automated method of analysis of apoptosis in FNAs to exploit the change at 24 hours for the purposes of patient management.

BODY:

Experimental methods:

Core-cut biopsies (14 gauge) and fine needle aspirates (FNAs) have been derived from consenting patients with primary breast cancer during their participation in one of a number of clinical trials of preoperative medical therapy:

- (i) A total of 300 patients randomised to receive either adjuvant or neoadjuvant MMT (mitoxantrone, methotrexate and tamoxifen).
- (ii) A total of 180 patients randomised to receive either neoadjuvant adriamycin + cyclophosphamide (AC) or epirubicin + cisplatin + 5-fluorouracil (ECF), with all receiving tamoxifen.
- (iii) A total of 50 patients receiving neoadjuvant ECF.
- (iv) A total of 50 postmenopausal patients receiving neoadjuvant tamoxifen.

Core-cut biopsies were fixed in formalin and embedded in paraffin-wax. Histological sections (3 μ m) were taken onto charged slides. FNAs were suspended in tissue culture medium (minimum essential medium + 25 mM HEPES) and portions were taken for cytopsin preparation and flow cytometry. Cytopins were stored at -70°C. Aliquots for flow cytometry were fixed firstly in 1% paraformaldehyde/PBS and then in 70% ethanol and stored at -20°C. Sections were stained using the MIB-1 antibody to the Ki67 nuclear proliferation antigen and by the TUNEL technique for apoptosis (Ellis et al, 1998).

The cytopsin preparations were stained with the MIB-1 antibody (Makris et al, 1998). The aliquots for flow cytometry were subjected to propidium iodide staining and TUNEL assay as described in detail elsewhere (Dowsett et al, 1998).

Ki67 and apoptotic index (AI) were expressed as % ages of positively staining cells. For Ki67 at least 1,000 cells were counted and for AI at least 3,000, when sections were used. FNA estimates of Ki67 required at least 100 cells.

Results:

- (i) 24-hour core cut study (ECF or AC treatment)

Our original series of 19 pairs of core cuts showed an overall mean increase of in AI from a medium 0.47% to 1.02% over the first 24 hours chemotherapy. A

further 22 pairs showed a significant increase by a mean increase of 151% (Fig 1) 17 of these increased by at least 50% which is our previously established limit for statistically significant change (Ellis et al, 1997). A breakdown of clinical response will be made after the completed follow-up of a total of 50 patients, but this is likely to be compromised by the addition of tamoxifen which may influence response but is not likely to influence apoptosis within 24 hours. A further observation has, however, confirmed the biological value of these measurements: it has been found that c-erb B2 positive tumours (which in some clinical studies show a poor response to chemotherapy) have a significantly poorer chance of having a significant increase in apoptosis than the c-erb B2 negative group: 30 c-erb B2 negative tumours showed a mean increase in AI of 245% while 9 c-erb B2 positive tumours showed an increase of only 34%. This work will be presented at the 1998 San Antonio Breast Cancer Conference. This finding emphasises the value of these samples for the pathological assessment of parameters which may determine the apoptotic response to chemotherapy (such as p53 or bcl-2 family status).

The changes in proliferation which occurred in these same samples are shown in Figure 2. Only 9/35 samples showed a >50% decrease in Ki67 but overall there was a fall from a median value from 28.5% pretreatment to 16.9% after 24 hours. This is the first time that changes in proliferation of this type have been shown to occur so early after starting chemotherapy. We therefore have a set of samples within which we can explore the involvement of regulators of the cell cycle such as p21^{cip1} or p27^{kip1} in this biological response. We feel that we should examine the possibility that changes in these regulators might be better predictive indices of clinical response than changes in proliferation and apoptosis themselves.

(ii) FNA 2/3 week proliferation study:

The results from our studies of Ki67 in FNAs taken before and after 14-21d of MM chemotherapy, MMT chemoendocrine therapy and tamoxifen therapy are shown in Figure 3 (a), (b) and (c). In all 3 sets it can be seen that there was a significant fall in the responders to therapy but no fall in the responders. The change was, however, not consistent between patients in the 2 groups. The utility of this measurement will be evaluated by assessing the predictive power for response or resistance. In future studies we will collect samples at both 24 hours and 21 days to assess which of these might provide the most reliable predictive material. (Collections from 8 such patients are already available). Apoptosis was not measured in these FNA samples because of the unsuitability of cytospin

preparations for such measurement and the continuing development of the automated technology (see below).

(iii) Development of an automated method for FNAs

(a) Initial studies using FNAs from surgical samples and flow cytometry:

For our initial studies, we took multiple fine needle aspirates from primary breast carcinomas after their surgical removal. The aspirates from each tumour were pooled to give a high number of cells for study. Apoptotic cells were labelled using the TUNEL assay. The nuclei of all the cells in the preparation were labelled with propidium iodide and green fluorescence (apoptotic cells) was displayed against red fluorescence (DNA). The identification of the green positive cells was confirmed by cell sorting followed by fluorescence microscopy.

In many samples, the number of apoptotic cells was less than 1% while the intensity of staining varied from dim to very bright. The delineation between positive and negative in the DNA histogram was therefore critical (The negative cells always exhibit some fluorescence due to autofluorescence and some non-specific staining). Correct delineation depends on the accurate identification of the negative population. We investigated two approaches to tackling this problem.

1. We split samples in two and performed the TUNEL assay with and without the key enzyme, terminal deoxynucleotidyl transferase. In the absence of the enzyme, all the cells are negative, which should allow positive cells to be accurately defined. However, it was observed that, in some samples, the negative cells (the bulk of the population) had slight positivity which suggested that the DNA of some of the non-apoptotic cells contained a low number of strand breaks. The effect meant that we could not use this method to define the boundary between negative and positive cells.
2. The green fluorescence was recorded using logarithmic amplification. We observed that generally the negative cells showed a log normal distribution, that is, on a logarithmic scale, the distribution was close to normal. We measured the mean fluorescence of the negative cells and the standard deviation (SD) of the distribution. We then took the boundary between negative and positive as being the mean plus $3 \times \text{SD}$. For a normal distribution, using this criterion, 0.013% negative cells would fall in the positive region. The choice of $3 \times \text{SD}$

was made by experimenting with a model system which contained well-defined positive and negative populations. The percentage of apoptotic cells recorded by flow cytometry using this approach gave an acceptable correlation with the percentage of apoptotic cells observed in a tissue section from the same tumour (Fig 4). This method was adapted for all future work (Dowsett et al, 1998).

(b) Further validation work using FNAs from patients and flow cytometry:

A series of FNAs were taken from patients with primary breast carcinomas and stained for flow cytometry using the TUNEL assay. Three problems were identified.

1. In a few samples there were large numbers of red blood cells which clumped to each other and to the epithelial cells during fixation and interfered with the staining reaction. Standard methods for lysing red blood cells also lysed small numbers of epithelial cells.
2. The minimum number of cells needed for staining was 100,000. For optimum staining, a larger number of cells is preferred but is frequently not available.
3. Because of the small number of positive cells present in most samples, confirmation of the identification of these cells as apoptotic is necessary for validity purposes. Cell sorting followed by fluorescence microscopy, while possible, is time-consuming.

It was therefore concluded that, while flow cytometry remained a possible option, there were substantial difficulties and we should therefore explore alternative technologies.

(c) Laser scanning cytometry (LSC):

LSC is a complementary technology to flow cytometry. In the flow cytometer, cells in suspension, move past a static laser beam. In the LSC, the cells are static, on a microscope slide, and are scanned by a moving laser beam. In both instruments, fluorescences from single cells are recorded. For our purposes, the LSC has two advantages.

1. It uses fewer cells; cells on a cytospin can be stained and recorded.
2. The position of each cell on a slide is recorded. Cells with given fluorescence properties can be relocated and observed under the microscope. In an apoptosis assay, positive cells can be quickly examined and their identity confirmed. Therefore fluorescence cut-

off points for apoptotic cells can be derived or applied with confidence.

We have applied for a supplementary grant to the US Army Research and Material Command for 50% funding towards such an instrument and this application is under consideration. In the meantime we have started initial studies on an instrument purchased by the Imperial Cancer Research Fund, London, using cultured cells. We have successfully identified apoptotic cells stained on a microscope slide using the TUNEL assay (Figure 5). For this preliminary work we used HL60 cells (a human promyelocytic cell line) treated with camptothecin to induce apoptosis.

We will continue this preliminary work using a breast cancer cell line, MCF7. When we have established the correct conditions for preparing (fixation conditions, etc.) and staining the cells, we will evaluate the use of this technology for studying FNAs from human breast carcinomas.

Studies in progress:

Further collections of core-cuts and FNAs are being made from patients in 3 neoadjuvant studies for assessing the predictive power of these assessments:

- (i) Chemotherapy: Navelbine + mitoxantrone vs AC (in 200 patients) began August'98; to complete beginning 2000. At least 50% of these patients will not receive tamoxifen before surgery. It will therefore be possible to directly relate clinical response to pathological changes, which has not been possible in our earlier studies.
- (ii) Endocrine therapy:
 - (a) vorozole vs. tamoxifen (50 patients) completed October'98.
 - (b) anastrozole vs. tamoxifen vs. tamoxifen + anastrozole (150 patients) started April'98; to complete late'99.

Pretreatment, 2 week, 6 week [(b) only] and 3 month core cut & FNA samples will be assessed for proliferative and apoptotic changes.

CONCLUSIONS:

Our present studies have confirmed the changes in apoptosis which occur after 24 hours chemotherapy. A prospective study in which the relationship with response can be assessed is in place.

Changes noted to occur in proliferation after 24 hours will allow exploration of the biological determinants of the changes in both proliferation and apoptosis in breast carcinomas.

Changes in proliferation at 2-3 weeks are related to response, but their predictive power remains to be determined.

Flow cytometry may be difficult to apply to measurements of apoptosis in breast carcinomas but early data from LSC is encouraging and will be pursued.

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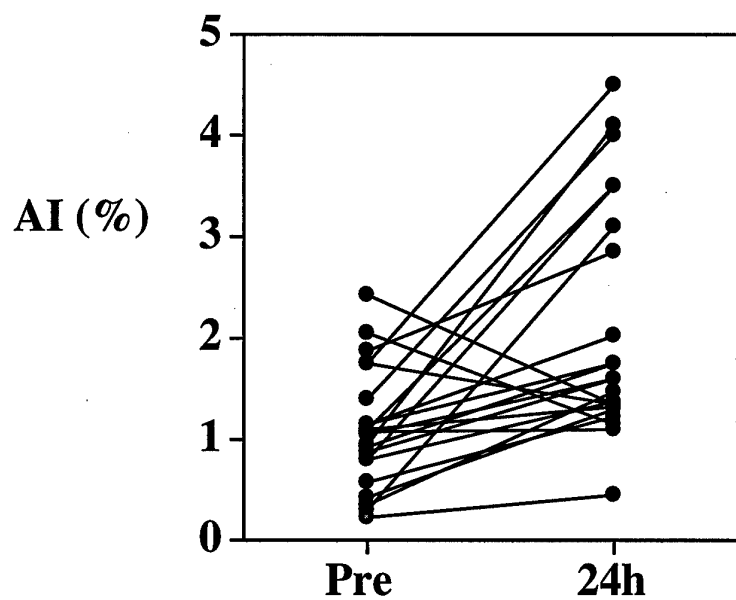


Figure 1: Increase in apoptosis in breast carcinomas after 24-hours chemotherapy confirms the relationship which we observed in our earlier series (Ellis et al, 1997)

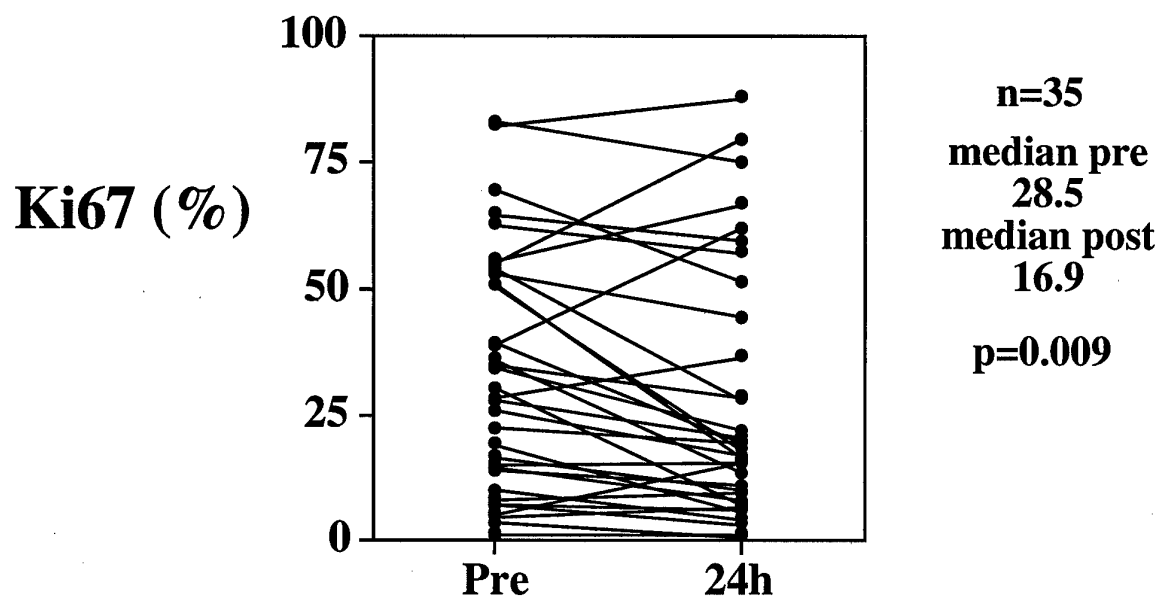


Figure 2: Decrease in proliferation in breast carcinomas from 35 patients after 24-hours chemotherapy. This is the first study to demonstrate this early change.

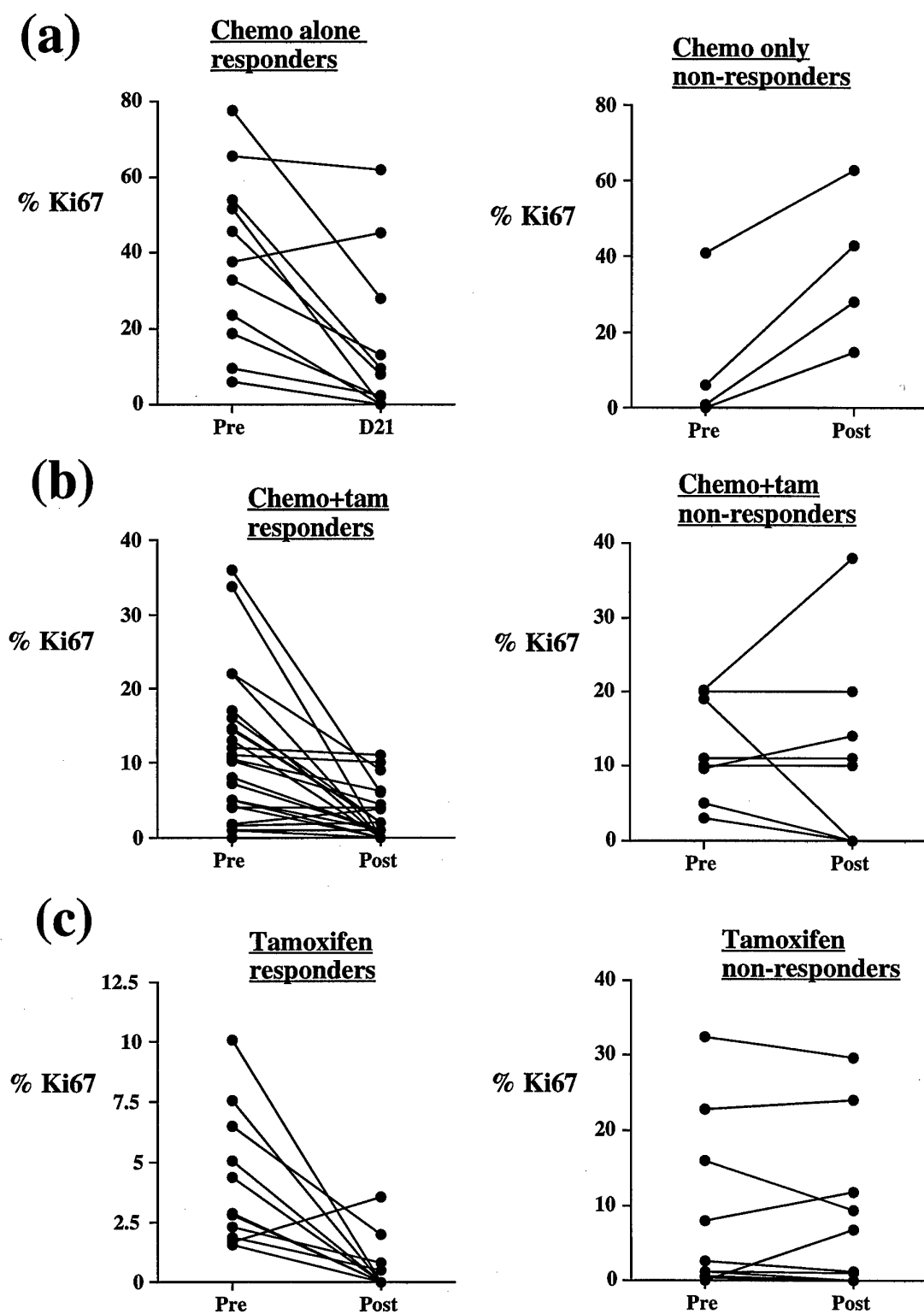


Figure 3: Significant reductions in proliferation in responders but non-responders to (a) chemotherapy, (b) chemoendocrine therapy and (c) tamoxifen therapy

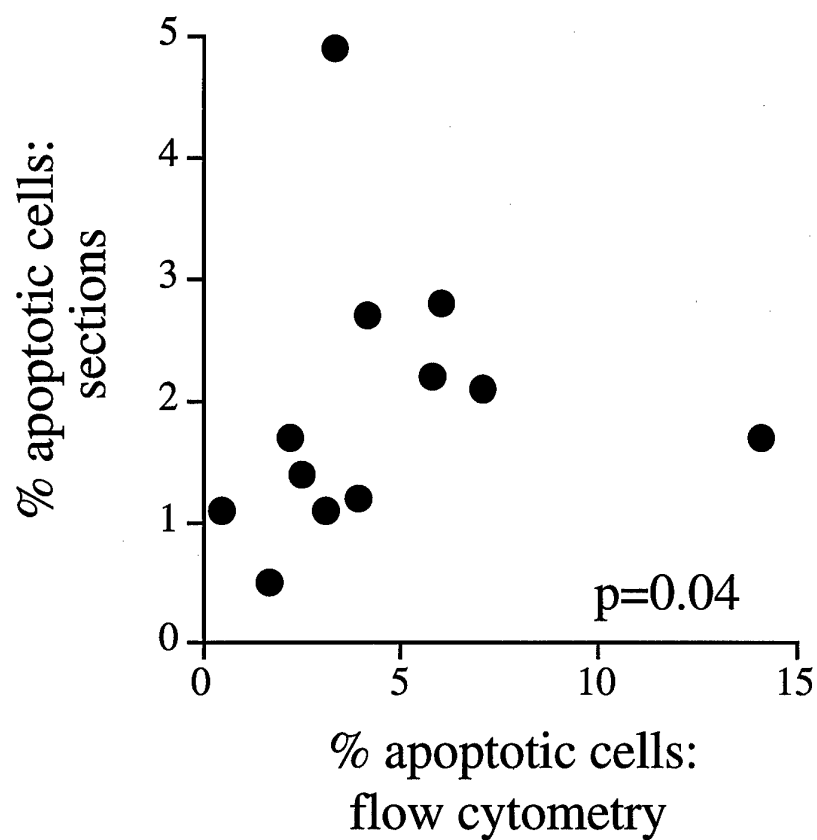


Figure 4: Relationship between apoptosis as measured by TUNEL in FNAs with flow cytometry and on sections by conventional microscopy.

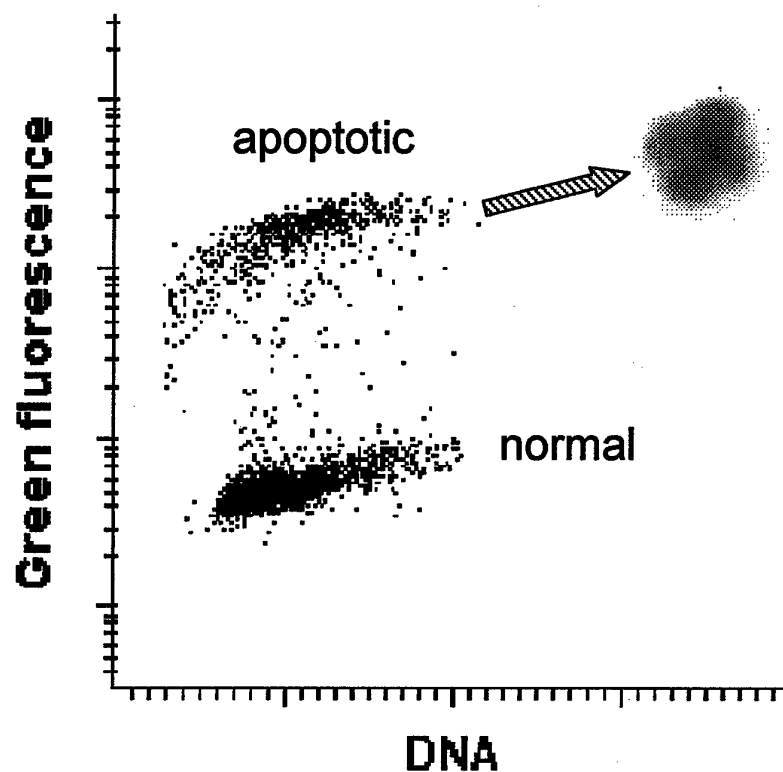


Figure5 . HL60 cells treated with camptothecin to induce apoptosis. The cells were labelled using the TUNEL assay and their fluorescence recorded using a laser scanning cytometer. The insert shows the the fluorescent image of the nucleus of a cell which was relocated from within the apoptotic cluster.